Fluorimetric multiparameter cell assay at the single cell level fabricated by optical tweezers

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Abstract A fluorimetric multi-parameter cell sensor at the single cell level is presented which makes it possible to observe the physiological behavior of different cell lines, different physiological parameters, and statistical data at the same time. Different cell types were immobilized at predefined positions with high accuracy using optical tweezers and adhesion promoting surface layers. The process is applicable to both adherent and non-adherent cells. Coating of the immobilization area with mussel adhesive protein was shown to be essential for the process. Intracellular proton and calcium concentrations in different cell classes were simultaneously imaged and the specific activation of T lymphocytes was demonstrated. This method should be especially useful for drug screening due to the small sample volume and high information density.

Key words: Optical tweezers; Cellular screening; Single cell technique; Cell sensor; Intracellular calcium

1. Introduction

Drug screening is the examination of the effects of new substances on isolated receptors, cell cultures, isolated organs and laboratory animals in serial tests. It is one of the most important steps during the development of new drugs. During the last years, especially high-throughput screening, a preclinical process by which large quantities of potential drugs can be tested automatically, became an important tool in finding new drugs [1].

Fluorimetric cellular microassays are a valuable tool for the screening of drugs, since they allow a sensitive evaluation of the influence of a substance on cell proliferation, mitochondrial activity or cell viability [2,3]. Hence, the response of a cellular biosensor to an applied drug represents its whole physiological effect compared to conventional approaches like affinity methods, in which the binding to only one specific receptor is crucial [4]. But knowledge of the physiological effects of drugs is essential for their application.

However, cellular microsensors are difficult to adapt to modern strategies of drug development where only small quantities of substances are available, in particular when microbeads are used for the synthesis of chemical libraries having a loading capacity of only a few nanomoles [5]. Therefore, a device featuring the possibility of drug screening at the single cell level would be very useful.

We have therefore developed a method for defining the distribution of different cell lines on a surface and addressing them separately resulting in a high information density. So far, approaches for the fabrication of organized cellular assemblies on surfaces have utilized mainly photolithographic techniques [6] or microcontact printing [7]. While these approaches offer the possibility of patterning the adhesion of cells on rather large areas, they cannot be used to define the distribution of more than one cell line on a surface. Hence, we examined the applicability of optical tweezers, a powerful optical tool firstly introduced by A. Ashkin [8]. The root of optical tweezers is a laser beam, highly focused by a microscope objective. Such a device allows one to trap, transport and isolate small objects in the range of micrometers like single cells by applying optical forces [9–12]. We employed the optical tweezers for the fabrication of cell patterns. These organized cellular assemblies fixed to solid supports can be used for highly efficient and miniaturized drug screening.

2. Materials and methods

2.1. Optical tweezers

The optical tweezers set-up consists of a Nd:YAG laser (1064 nm, 350 mW, Adlas, Lübeck Germany) coupled to a Zeiss Axiovert microscope (Zeiss Oberkochen Germany). The use of an Ultrafluar microscope objective (100 N.A. 1.25, gly., Zeiss) allows comfortable cell transportation over large distances thanks to its rather high working distance.

2.2. Manipulation mask

A silicone chamber was fabricated by polymerization of silicone rubber in a matrix such that a central chamber (9 mm^2) was linked to four chambers of the same size via 0.2 mm wide channels (Fig. 1). The silicone chamber was gently pressed onto a coverslip which had been hydrophobized with octadecyltrichlorosilane (OTS) in order to seal up the system.

2.3. Immobilization process

A solution of 0.4 μg mussel adhesive protein (MAP, also known as Cell-Tak, Becton-Dickinson) in 10 μl 0.2 M $NaHCO_3$ was placed in the central chamber. MAP is a formulation of polyphenolic proteins which are the key components of the anchoring glue secreted by the marine mussel *Mytilus edulis* [13]. The usefulness of MAP for the immobilization of non-adherent cells for fluorimetric studies has been demonstrated previously [14]. After 30 min the chamber was washed twice with HEPES buffer. Note that during the coating process the aqueous solution does not penetrate the channels because of its highly hydrophobic character. Thereupon, 20 μl of a solution of 1% of the copolymer Pluronic F 68 (poly(propyleneoxide)poly(ethyleneoxide) [PPO-PEO], Sigma) in HEPES buffer was added to each of the four other chambers for a further 30 min. Adsorption of the polymer in the channels resulted in the opening of the channels. Before adding cells, the chambers were rinsed twice with HEPES buffer. Adsorption via the PPO segment results in a PEO-terminated surface which almost totally prevents cells from adhering [15]. A few microliters of the corresponding cell suspensions were carefully injected into the Pluronic coated chambers.

2.4. Cell culture

The human T cells JMP and the mouse myeloma cells SP2 were cultivated at 37°C, 10% CO_2 and 95% humidity in 250 ml flasks

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(Nunclon, Nunc). The medium for both cell lines consisted of RPMI 1640, 10% heat-inactivated fetal calf serum and 1% 0.2 M glutamine. Medium was changed every 3–4 days.

Human peripheral blood mononuclear cells (PBMC) were isolated by centrifugation in Ficoll-Hypaque. Further isolation of small lymphocytes was achieved by morphological discrimination under the microscope using optical tweezers.

The pheochromocytoma cells PC12 were also cultivated at 37°C, 10% CO_2 and 95% humidity in dishes, 15 mm in diameter (Nunclon, Nunc). The culturing medium consisted of DMEM (Dulbecco's modified Eagle's medium), 10% heat-inactivated fetal calf serum, 5% heat-inactivated horse serum, 1% 0.2 M glutamine and 1% penicillin/streptomycin. Monolayers were harvested by repeated gentle streaming of the conditioned medium over the growth surface. Then, the resulting cell suspension was centrifuged, the medium was removed by suction and cells were subcultured at a split rate of one to five.

3. Results

In order to build cell patterns, firstly the cells were injected in the Pluronic-coated chambers. For estimating the force of attraction between the cell and the Pluronic surface, we determined the minimal laser power for trapping lymphocytes and compared this value with the laser power necessary for detaching cells. The force on the cell was taken to be the difference between the gravitational and buoyant forces, given by $F_g - F_b = V_c g\,(\rho_c - \gamma)$, where V_c is the volume of the cell, ρ_c is the density of the cell (for lymphocytes $\rho_c = 1.04$–1.09 g/cm [16]), g is the acceleration due to gravity and γ is the specific weight of the surrounding fluid ($\gamma = 1.02$ g/cm for HEPES buffer). For lymphocytes with an average diameter of 14 μm this results in a trapping force between 0.23 pN and 0.79 pN. Since laser powers corresponding to about twice as high were needed to detach the cells from the Pluronic surface, the average force to detach a lymphocyte from a Pluronic surface is between 0.46 pN and 1.58 pN. In particular, only two of a total number of 30 lymphocytes (6.6%) could be detached from an OTS-derivatized surface, the adsorption of Pluronic reduced cell adhesion significantly: 26 of a total number of 30 cells could be detached with an initial laser power ranging from 35 to 105 mW. These laser powers correspond to approximate forces of 1.5–4.5 pN. Only four out of 30 cells could not be detached from the Pluronic surface.

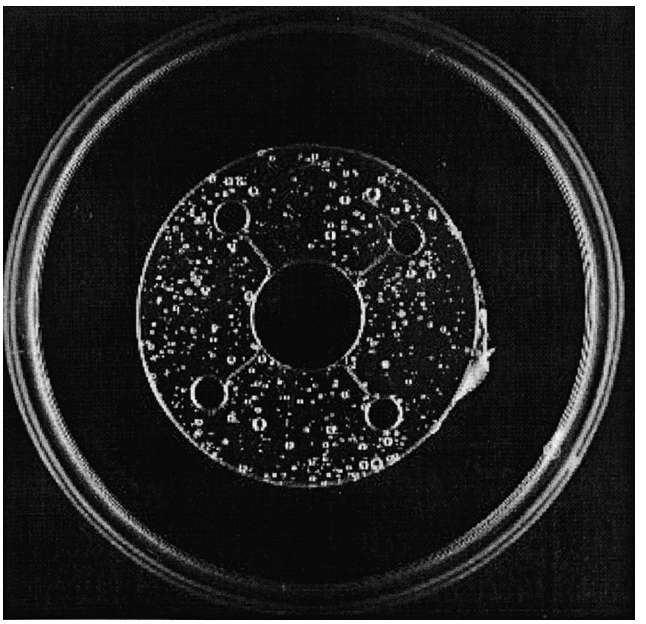

Fig. 1. A photograph of the silicone mask in which the experiments were performed. The satellite cavities were coated with the anti-adhesive Pluronic. From there, cells were transported via the channels with the optical tweezers to the central chamber, coated with the cell-adhesive MAP.

For positioning, the cells were moved from the Pluronic-coated chamber through the channels and immobilized at pre-

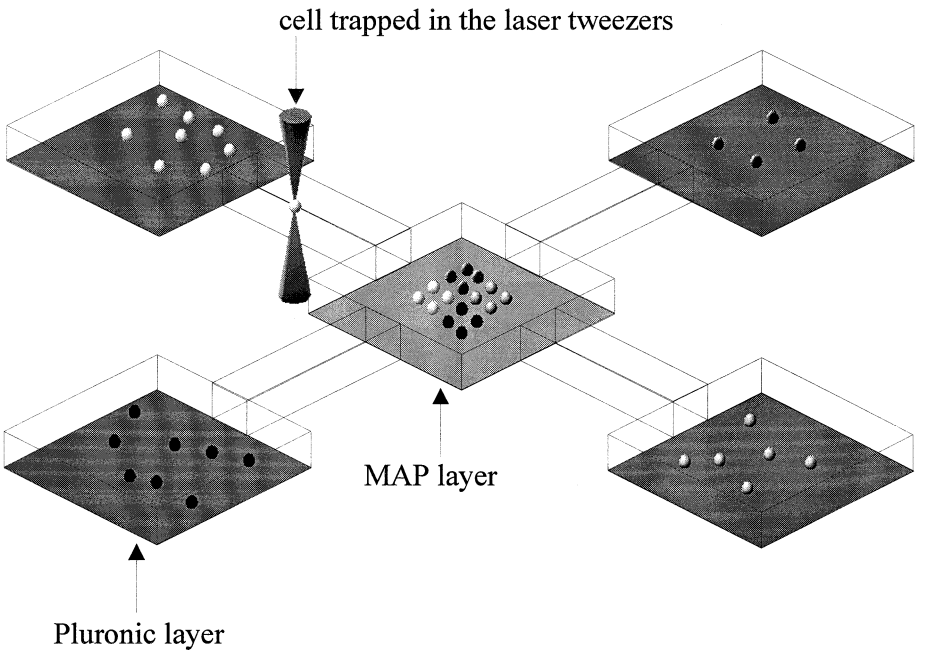

Fig. 2. A schematic drawing of the principle of our multiparameter cell assay. Cells loaded with different fluorescent dyes are injected in the satellite cavities of a silicone chamber, coated with the non-adhesive Pluronic. From there, the cells are transported to the central chamber with the optical tweezers and are immobilized on the MAP layer.

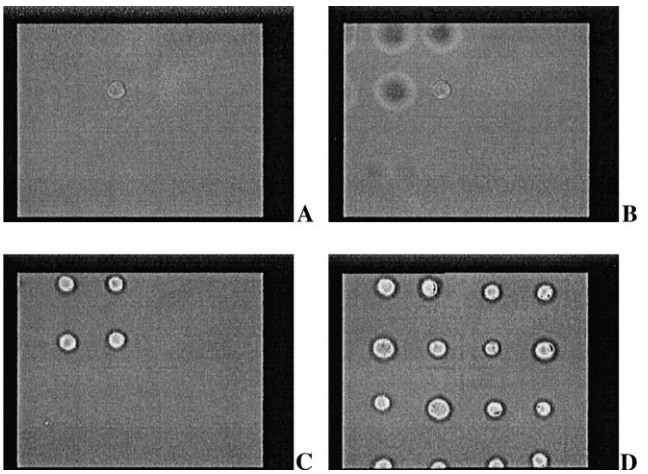

Fig. 3. Sequence of photographs of the immobilization procedure. A lymphocyte is trapped with the optical tweezers and transported to the defined position for immobilization (A and B). At this position the cell is pressed onto the substrate for a few seconds (C) before the cell is immobilized at an exact position within an organized assembly of cells (D).

defined coordinates in the MAP-coated chamber (Fig. 2). Immediately (<3 s) after contact with the surface the cells attached in such a way that the optical tweezers no longer exerted sufficient force to detach them. Furthermore, the cells remained exactly at their assigned position (Fig. 3). The presence of bovine serum albumin prevented this rapid immobilization process. Also, we found that neither pure electrostatic (poly-L-lysine) nor integrin-receptor interactions (PC12 cells on fibronectin) were sufficient for accurate positioning.

As one cell type to be immobilized, small lymphocytes directly isolated from healthy subjects were chosen. Lymphocytes are particularly attractive for drug screening applications since they possess the major cell defenses [17] and are easily accessible human cells. Their cell viability after the immobilization process was checked by incubating them with the viability and pH indicator BCECF. All cells remained vital, as shown by a strong fluorescence (Fig. 4).

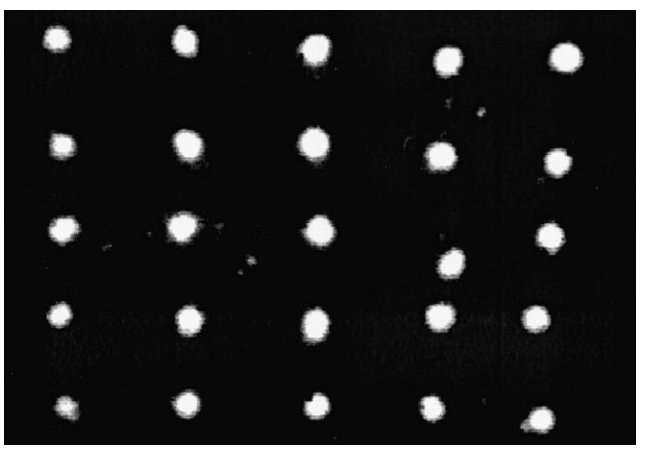

Fig. 4. Image of BCBCF-labeled lymphocytes recorded with a slow-scan CCD camera. A few microliters of human PBMC suspension, isolated from fresh, heparinized blood of healthy volunteers using Ficoll-Hypaque gradients, were added to one of the Pluronic chambers. Small lymphocytes were selected by morphological discrimination with the optical tweezers and immobilized in the MAP chamber. 0.2 μl of a solution of BCECF-AM ester (Molecular Probes) in DMSO was added and the fluorescence monitored for 30 min. All cells showed strong fluorescence with an excitation wavelength at 488 nm indicating their viability.

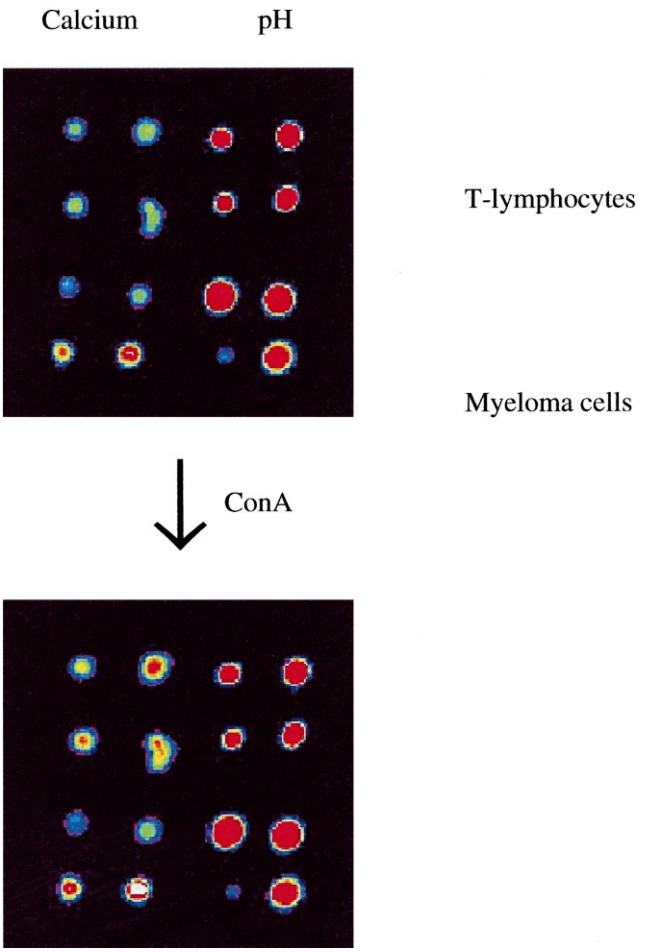

Fig. 5. Cells of four different species were immobilized in patterns using optical tweezers. Fluo 3-labeled cells (20 μM Fluo-3-AM incubated in HEPES buffer for 45 min) were immobilized on the left, BCECF-labeled cells (5 μM BCECF incubated in HEPES buffer for 20 min) were immobilized on the right. The eight top cells are human T cells (JMP), the bottom eight mouse myeloma cells (SP2). Here, the original 16 bit image is reduced to a 32 pseudocolor image for better visualization. The top part shows the cells before the addition of 0.4 μg succinyl-ConA (Sigma), the bottom part 9 min after the addition, when maximal Fluo-3 fluorescence was observed. All Fluo-3-labeled cells show a stronger fluorescence indicating an increased calcium concentration, whereas BCECF fluorescence remained nearly constant. However, the calcium rise is stronger in T cells.

In order to demonstrate that specific stimulation of the cells is still possible after the transportation and immobilization process, we loaded each of the four Pluronic chambers with cell types differing with respect to cell line and labeling. We incubated the T cell line JMP as well as the myeloma cell line SP2 with both the pH indicator BCECF and the calcium indicator Fluo-3. T cells can be activated by mitogenic lectins such as concanavalin A, which acts via the CD3/TcR complex [18]. SP2 cells do not express the CD3/TcR complex. Four cells of each group were positioned (Fig. 5). Fluo-3 and BCECF have very similar absorption and emission characteristics so that their fluorescence can be observed simultaneously without changing optical filters. Because the fluores-

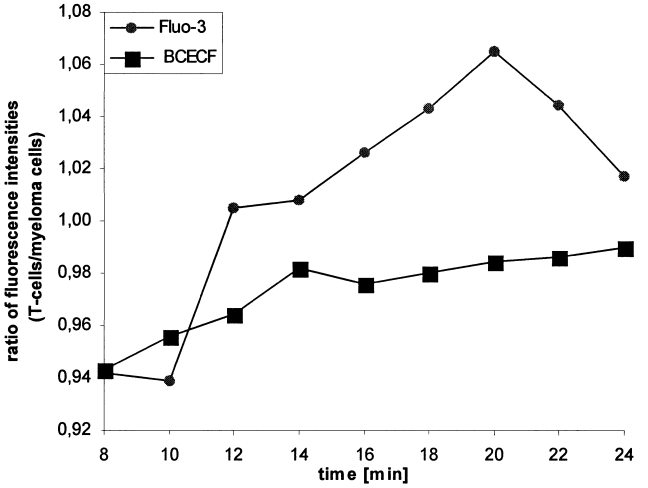

Fig. 6. Average mean intensities of each of the four groups of cells were obtained in NIH Image. In order to cancel out non-specific signals, the ratio of the mean intensities of T cell fluorescence and the mean intensities of myeloma cell fluorescence were calculated. Whereas the ratio of pH mean intensities remains nearly constant, a specific rise in T cell calcium concentration can be observed.

cence intensities of the indicators are rather different, we used a 16 bit camera for imaging. Fig. 5 shows a 32 pseudocolor image before and after addition of concanavalin A. While both BCECF-labeled T and myeloma cells show equally decreasing fluorescence intensities, a specific calcium rise can be observed in the T cells. Rationing the intensities of T and myeloma cells allows the canceling out of non-specific effects and thus indicates relative calcium or pH changes (Fig. 6). Again, the specific rise of intracellular calcium in the T cells can be observed.

4. Discussion

The optical tweezers-based manufactured cell patterns presented here combine the advantages of fluorimetric microtitration assays with the concept of whole-cell biosensors, which have gained interest as highly sensitive analytical devices for receptor screening applications [19–21] or as detectors in capillary electrophoresis [4,22]. Furthermore, since our method uses microscopy rather than a bulk technique for monitoring the cells, cell population heterogeneity can be accounted for. However, one should mention that at the present state of the art it is not possible to absolutely exclude effects of the immobilization process.

We believe that the fabrication of cell patterns as described here might become a standard laboratory method. No specialized equipment is needed except optical tweezers. Since only small laser powers are necessary (< 100 mW) for the immobilization process, rather inexpensive diode laser-based systems would be sufficient [23]. The number of cells can be chosen freely to fit the statistical demand of the specific assay, while it should not fall below a number sufficient to obtain comfortable statistical data. For industrial applications such as high-throughput screening the efficiency of the fabrication process needs to be increased to enable a mass production of cell patterns, e.g. for drug screening. This could be done by the use of multiple optical tweezers [10,24] and by decreasing the distances the cells are moved by microstructuring the device. Thus one could minimize the probe volume down to the submicroliter range and therefore help to miniaturize the screening assay.

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